- **Workflow:**
    - **Generating Competent Bacteria**
    - **Digestion & Ligation (place desired insert into plasmid vector)**
    - **Bacterial Transformation**
    - **Plate Growth: Select for bacteria that uptake the vector**
    - **Colony PCR (low-resolution genotyping)**
    - **Sanger Sequencing (high-resolution genotyping)**
    - **Overnight culture (of the inoculated, genotyped, and correct clones)**
    - **Or Bacterial Freezer Stock Generation**
    - **Plasmid Purification**
    - **Repeat**

- **Generating Competent Bacteria by CaCl2 method**
    - Strain: Invitrogen E.coli Stbl3
    - 100-200x dilution from frozen stock
    - Grow 2-3 hrs in exponential phase
    - Incubate on ice (to synchronize all cells, not mitosis phase)
    - Wash with pre-chilled 100 mM CaCl2, while on ice
    - Store competent bacteria at -80 C
    - Expected Yield: 5e6 to 2e7 transformed colonies/ ug of plasmid DNA
    - Stage 1: Preparation of Cells
        - Pick a single colony from a plate that has been incubated overnight at 37 C.
        - Inoculate that colony into 100-mL LB broth in a 1-L flask
        - Incubate at 37 C for 3 hours
        - Begin harvesting when OD 600 reaches 0.35
        - Transfer bacteria to 50-mL conical tubes on ice for 10 min
        - Centrifuge at 2700 g for 10 min at 4 C.
        - Decant supernatant. Invert tubes on a paper towel to dry out all supernatant
        - Resuspend with 30 mL of ice-cold MgCl2+CaCl2 solution (80 mM MgCl2, 20 mM CaCl2). Vortex.
        - Centrifuge at 2700 g for 10 min at 4 C.
        - Decant supernatant. Invert tubes on a paper towel to dry out all supernatant
        - Resuspend with 4 mL of ice-cold 0.1 M CaCl2 (2 mL per 50 mL of original culture). Vortex.
        - Dispense the competent bacteria into aliquots and store at -80C

- **Generation of Insert** (Oligo can be encoding for gRNA to be inserted into H3 or pXPR_003 plasmid vectors)
    - Go to UCSC Genome Browser
        - Type in Gene Name that you want to Knock-Out
        - Scroll down to “Genes and Gene Prediction” section

        - Display “CRISPR Targets” in “full”
        - The green sequences are better NGG
    - Order oligo synthesis from IDT
        - Find 20210621_gRNA_design.xlsx to use as template
        - Add "g" to 5’ end of TOP strand
        - Complete TOP strand: cacc+g+seq
        - Reverse Complement of TOP to get BOTTOM
        - Add “aaac” to 5’ end of BOTTOM strand
        - Name the ssDNA oligo to be purchased
    - Liquidify oligos
        - 100 uM per oligo: 10 nmol mixed in 100 uL dw
        - Formula for oligos mixture in PCR tube strip:
            - 5 uL TOP strand
            - 5 uL BOTTOM strand
    - Phosphorylation of 5’ end of oligo (synthetic oligos do not have 5’ phosphate)
        - Formula in PCR tube strip:
            - 1 uL T4 DNA ligase 10X Buffer
            - 0.5 uL PNK Enzyme (polynucleotide kinase)
            - 6.5 uL dw
            - (2) uL oligos mixture
        - Put in PCR machine:
            - 37 C for 30 min
            - 95 C for 5 min
            - 28 Cycles cooldown
                - -2.5 C decrement per cycle
                - 30 sec per cycle
        - Before ligation, dilute the phosphorylated oligos 10X: 90 uL dw + 10 uL oligos mixture

- **Digestion & Ligation**: Place desired insert into vector
    - **Digestion of vector (linearization)**
    - **Ligation**
        - Formula in PCR tube strip:
            - 1 uL T4 DNA ligase
            - 1 uL T4 DNA ligase 10X Buffer
            - 1-2 uL linearized vector H3 (enough to get 50 ng of vector per rxn)
            - 2 uL phosphorylated oligos mixture
            - Top off with dw to get final volume of 10 uL per rxn
        - Mix by pipetting up and down or tap tap
        - Incubation
            - Sticky ends: room temperature for 10 min (or 16 C overnight)
            - Blunt ends: room temperature for 2 hours (or 16 C overnight)
        - Heat inactivate at 65 C for 10 min

        - Chill on ice
- **Bacterial Transformation by Heat-shock Method**
    - Get aliquots of competent E coli Stbl3 bacteria from -80 C. Thaw on ice for 15 min.
    - Distribute in Each PCR tube: 50 uL bacteria + 5 uL ligation product (plasmid DNA with insert) (10% ratio of DNA:bacteria)
    - Vortex to mix. Slight spin down with bench-top microfuge.
    - Incubate mixture on ice for 30 min.
    - Heat shock @ 42 C for 45 sec.
    - Incubate on ice for 2 min.
    - In 2-mL Eppendorf tubes, add 400 uL of pre-warmed SOC media per tube.
    - Transfer transformed bacteria into these SOC-filled Eppen tubes.
    - Keep in warm room on shaker for 2 hours for regeneration
    - Using a P1000 tip, suck up the bacteria and spread on to Cb+Lb petri dishes.
    - Leave dishes in warm room overnight.
    - Notes:
        - SOC media bought from Boston Bioproducts
        - Recipe

- **Colony PCR (low-resolution genotyping)**
    - Add 50-75 uL Lb+Cb broth per well to 96-well PCR plate (leave out the edges)
    - Pick 3 colonies per agar+Cb dish (triplicates) with sterile P20 tips
        - These colonies have picked up the plasmid
    - Incubate 1 hour in warm room 37 C
    - Formula in 384-well plate:
        - 12.5 uL ThermoScientific PCR 2X MM
        - 11 uL dw
        - 1 uL of vector-specific Primer Mix (10 uM each from 80dw+10F+10R mixture)
          *OV267-F,OV268-R for pLentiCRISPRV2
        - DNA template (heat lysis of bacteria)
        - Total Volume: 25 uL
    - Keep the 96-well PCR plate, if PCR genotype is fine → use it as stock
    - Touchdown PCR Phire 1kb TD65 check machine
        - 1st 10 cycles: 98-65-72
        - 2nd 10 cycles: 98-60-72
            - Non-permissive 65 C annealing temperature → permissive 60 C annealing temperature
            - Initially, only very specific hybridizations are allowed to occur
        - Temperature increment: -0.5 C per cycle
        - Primer design:
            - Spanning U6 promoter to after the gRNA scaffold (370 bp region)

- **Gel Electrophoresis (to verify we have product)**
  - See agarose gel making protocol
- **Sanger Sequencing (high-resolution genotyping)**
  - Send pre-mixed template + primer
  - Diluted primer Formula
    - 5 uL FORWARD primer
    - 255 uL dw
    - Total Volume 300 uL
  - Formula of sample to submit to Genewiz
    - 13 uL diluted F primer
    - 2 uL PCR product
  - GeneWiz Instructions
    - PCR Product (Purified)
    - Service Type: Premix (Primer + Template)
    - "Purification": "Enzymatic"
    - Put in the bag a note: the barcode, # of samples submitted, Sanger Sequencing

- **Overnight culture (of the inoculated, genotyped, and correct clones)**
  - Prepare 50 mL Lb + Cb in 1 50-mL conical tube for every correct clone.
  - Add ~75 uL (all) of the regenerated clone from the 96-well PCR plate to each conical tube
  - Vortex.
  - Split into 2 50-mL conical tubes (duplicates)
  - Overnight 37 C shaker for 14-16 hours (no more or less)

- **Stock Generation by Freezing**
  - From overnight culture of the **inoculated, genotyped, and Sanger-correct clones**
  - Add 1m L overnight growth to 2-mL cryovial
    - 1.5-mL Eppendorf tubes can snap open at -80
  - Top off with 1 mL 50% glycerol (dilute 1:1 beforehand with dw)
  - Store at -80 C

- **Plasmid Purification by QIAgen PlasmidPrep Midi Plus Kit; high-yield protocol**

Table 3. Maximum recommended culture volumes

Copy number	Standard protocol	High-yield protocol
High-copy plasmid*	20–25 ml	25–35 ml
Low-copy plasmid*†	50 ml	–

* For high-copy plasmids, expected yields are ▲100–200 µg for the QIAGEN Plasmid *Plus* Midi Kit using the standard protocol and ●150–250 µg for the QIAGEN Plasmid *Plus* Midi using the high-yield protocol. For low-copy plasmids, expected yields are ▲30–100 µg for the QIAGEN Plasmid *Plus* Midi Kit using these culture values with the standard protocol.

  - Collect bacteria from overnight culture, suspension total volume 50 mL

- Centrifuge 50-mL Falcon tubes for 10min at 3000g, discard supernatant
- Vortex Falcon tubes to break pellets.
- Add 4 mL P1 Lysis Buffer (TEG, hypo-osmotic lysis)
    - Shake, vortex or pipet until no cell clump. Wait 3-4 min
- Add 4 mL P2 Buffer (blue, SDS detergent, basic pH) to denature bacterial & plasmid DNA
    - Carefully flip tubes 10x to precipitate bacterial gDNA, protein, lipid
    - Wait 3-4 min
    - viscous lysate, mix until homogenize; the solution should be blue
- Add 4 mL S3 binding buffer (acetic acid) to quench
    - Carefully flip tubes 10x to mix till the blue color disappear
    - white precipitate = gDNA, proteins, cell debris, SDS
    - Do not wait
- Centrifuge for 5 min at 4500g, Prepare the vacuum manifold and QIAGEN spin columns
- Insert the plunger into the QIA filter cartridge and filter the cell lysate into a new tube
  *filter into the extension tube directly above the spin column
- Add 2 mL BB buffer to the cleared lysate, invert 4-6 times
  *or add BB to the extension tube
- Transfer lysate to the spin column
- Switch on the vacuum source ~300 mbar
- Wash DNA with 0.7 mL ETR buffer, switch on vacuum then switch off
- Wash DNA with 0.7 mL PE buffer, switch on vacuum then switch off
- Put the column inside a 1.5 mL tube, centrifuge 10,000 g x 1 min to remove residual wash buffer
- Place a column into a new 1.5 mL tube; elute DNA with 200 uL EB buffer or water to the center of a spin column, wait 1 mins and centrifuge 10,000g x 1 min
  *Elute with 100 uL if high concentration needed
  *DNA stored in water may degrade over time
- Store DNA at -20*c